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Synthesis of Bifunctional Integrin-Binding Peptides Containing PEG Spacers of Defined Length for Non-Viral Gene Delivery

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Improving the buffer and serum stability of non-viral gene delivery vectors, and increasing their circulation time in vivo, is an important focus of current research in gene therapy. The most successful strategies to date have involved shielding the complexes with large polydisperse PEG adducts. However, this approach is accompanied by a fall in transfection efficiency. In this paper we describe the solid-phase synthesis of a series of bifunctional peptides bearing short PEG spacers of defined structure as components of lipopolyplex gene delivery vectors. Short, high-yielding routes to a series of PEG-amino acids are described: these PEG-amino acids can be used in varying combinations to afford bifunctional peptides with varying lengths of PEG spacers by using standard solid-phase synthesis techniques. A series of lipopolyplexes were formulated using these bifunctional peptides, and their transfection properties assessed. Dynamic light scattering measurements on the complex with the best transfection properties showed that in phosphate-buffered saline this complex was considerably more stable, and aggregated more slowly, than a complex formulated using a similar peptide lacking the short PEG spacer.

Introduction

Non-viral gene delivery systems typically involve the condensation of plasmid DNA with either cationic polymers (e.g., polylysine peptides) to form polyplexes,[1] cationic lipids to afford lipoplexes,[2] or both lipids and peptides to give lipopolyplexes.[3–7] A recurrent problem with these positively charged complexes is that they are bound by plasma proteins and therefore quickly cleared by the reticuloendothelial system (RES), which limits their circulation time in vivo.[8] In addition, such formulations are unstable in buffer and serum, forming aggregates and/or looser macromolecular complexes. Several approaches to improve the in vivo stabilisation and serum shielding of non-viral vectors have been attempted,[9–11] including shielding the cationic complexes with high molecular weight polymeric PEG. With some lipoplex[12–14] and polyplex[15,16] systems such PEG shielding works well, resulting in both serum stability and efficient transfection. However, with many others[17–20] the vectors, although stable in the circulation, have greatly reduced transfection properties, either because of steric hindrance or failure of the DNA to be released from the cell following internalisation. In some cases PEG conjugation is even ineffective in stabilising the complex and protecting the DNA from in vivo degradation.[21] The effectiveness of PEG conjugation appears to depend on the cell type and, for modified cationic lipids, the length of the acyl chain.[22,23] Preparation of vectors with cleavable PEG moieties frequently improves transfection,[24,25] and, crucially, if a cell targeting group is accessible on the outside of the PEG coat, the transfection efficiencies are often restored.[26,27] It is notable, however, that all of these approaches have relied on the conjugation of large polymeric PEG units, and, possibly because of the synthetic effort involved, the role of vector components bearing short, defined PEG units in shielding non-viral vectors has not previously been investigated.

We have initiated a program of work to improve the stability, circulation time and transfection efficiencies of lipopolyplex vectors by synthesising the individual components with short PEG spacers of defined length and structure as an integral part of the component lipids and peptides. In this paper we report the synthesis of a series of bifunctional peptides that comprise a DNA-binding sequence (K_{16}) and a disulfide-bridged sequence binding to the $\alpha_5\beta_1$ integrin

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(CRRETAWAC) joined by a series of PEG-amino acids of defined length. As the optimum length of the PEG spacer required to simultaneously target the complex to the integrin and to shield the complex from unwanted interactions with plasma proteins and serum was not known, we have also devised short, high-yielding routes to PEG-amino acids **1**, **2** and **3** (Figure 1), which could be used, singly or in combination, to produce a range of different PEG-linked peptides.

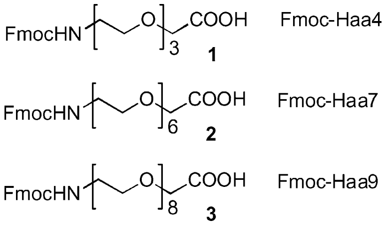


Figure 1. PEG-amino acids used in this study.

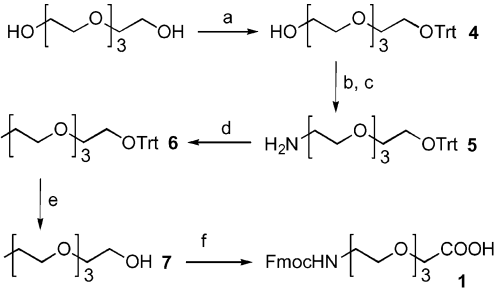
Results and Discussion

Synthesis of PEG-Amino Acids 1, 2 and 3

PEG-amino acids are valuable building blocks for the synthesis of tools for biological chemistry as they enable hydrophilic, flexible PEG spacers[28] to be readily inserted into biologically active peptides using standard techniques of solid-phase peptide synthesis (SPPS). It is desirable that synthetic approaches to these amino acids be high-yielding, require few purification steps (as difficulties are often encountered in the purification of PEG-containing moieties) and make use of readily available, low-cost starting materials such as tri- and tetraethylene glycol where possible.

A synthetic route to Fmoc-protected PEG-amino acid **1** (Haa4) for peptide synthesis was first reported by Boumrah et al.[29] who utilised a homologation of 2-(chloroethoxy)-ethoxyethanol with ethyl diazoacetate in the presence of $BF_3 \cdot OEt_2$ to build the desired tetraethylene glycol based ester, and converted the free hydroxy group to the azide and thence to the Fmoc-protected amino acid. This approach was recently modified by Thumshirn et al.[30] and Dekker et al.[31] to prepare the Fmoc-protected amino acid **2** (Fmoc-Haa7) from hexaethylene glycol. In all of these approaches, however, the overall reported yields were moderate, and the routes to **2** use the more expensive hexaethylene glycol. In addition, by following this approach, we encountered similar problems to those reported in the literature,[29] particularly with the final Fmoc protection of the amino acid, which has been reported in a number of systems to give poor yields and competing di-and tripeptide formation.[32]

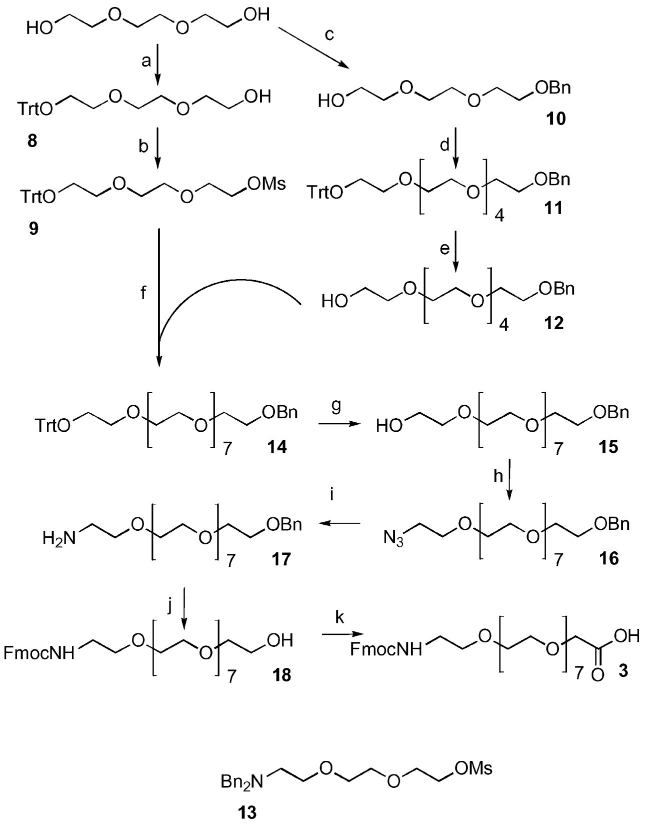
We therefore sought to develop a route to these amino acids from low-cost starting materials, avoiding some of the problems highlighted in the literature. To avoid the final Fmoc protection step, we aimed to synthesise the appropriate PEG-amino alcohol, protect the amino functionality and then oxidise it to give the carboxylic acid. For amino acid **1**, tetraethylene glycol was first monoprotected with the trityl group to give **4** (Scheme 1). This compound was then mesylated, treated with sodium azide and reduced under Staudinger conditions[33] to give the free amine **5**. This was readily protected to give **6** and the trityl group then removed to afford **7**. Careful choice of reagents was then necessary in order to effect oxidation to the carboxylic acid in the presence of both the Fmoc group and the PEG chain. Previous reports[34] had indicated that oxidation of PEG alcohols with sodium hypochlorite simultaneously resulted in extensive ether linkage cleavage, and we therefore carried out a series of test reactions on monobenzylated tetraethylene glycol under a variety of oxidising conditions. Oxidations with potassium dichromate,[35] potassium permanganate[36] and silver(II) oxide[37] were tested, but all caused either PEG-chain cleavage (determined by mass spectroscopy and NMR) or afforded the desired product in low yield. Oxidation with chromium trioxide in sulfuric and acetic acids[38] successfully converted the monobenzylated tetraethylene glycol into the desired acid in 84% yield without PEG-chain ether cleavage. However, when this method was attempted on the Fmoc-protected amino alcohol **7**, poor yields of partially deprotected amino acid were recovered. After further experimentation with various chromium oxidation methods, the Jones oxidation[39] proved satisfactory, affording **1** in 68% yield. The overall yield of the synthesis, over six steps, was 51%, and the reactions could be performed on a multigram scale.



Scheme 1. Synthesis of Fmoc-Haa4 (**1**). Reagents: a) TrtCl, Et_3N, DCM (95%); b) (i) MsCl, Et_3N, DCM; (ii) NaN_3, DMF (91%); c) PPh_3, H_2O, THF (96%); d) Fmoc-Cl, NMM, DCM (95%); e) (i) TFA, DCM, Et_3SiH; (ii) $NaHCO_3$ (aq.) (94%); f) CrO_3, 1.5 M H_2SO_4, acetone (68%).

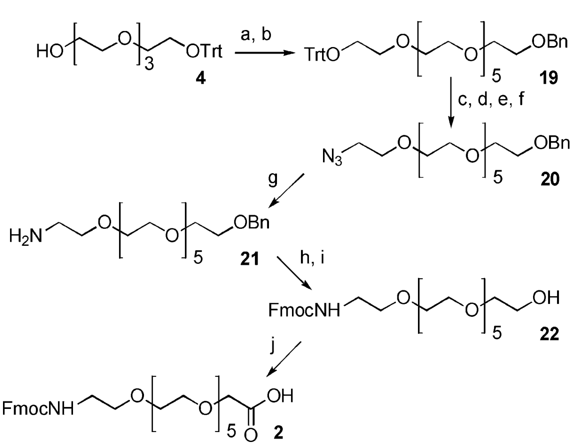
We were then able to generalise this approach to synthesise longer-chain PEG-amino acids. For amino acid **3** (Fmoc-Haa9), as nonaethylene glycol is not available, the PEG framework was constructed from triethylene glycol. We first synthesised the hexaethylene glycol skeleton from triethylene glycol as follows (Scheme 2). Monotritylation of an excess of triethylene glycol gave **8**, which was then mesylated to afford **9**. Similarly, monobenzylation of an excess of triethylene glycol in the presence of tetrabutylammonium bromide afforded **10**. Reaction of the mesylate **9** with the sodium salt of **10** gave the desired hexaethylene glycol unit

11, which was then detritylated under standard conditions to give **12**. We initially coupled this to the dibenzylamine **13**. However, removal of the *O*-benzyl protecting group from the resulting tribenzylated material proved impossible; hydrogenation catalysts were poisoned by the primary amino group,[40] whereas dissolving metal reduction, although successful in removing all three protecting groups, was not satisfactory as it was then impossible to isolate and purify the resulting highly water-soluble amino-PEG9 alcohol. Instead, mesylate **9** was coupled to the sodium salt of **12** to afford **14**, which was detritylated (to **15**) and converted into the azide **16** using the conditions previously described. Again, simultaneous azide hydrogenation and *O*-debenzylation using a variety of methods[41–43] was unsuccessful, with only partial removal of the *O*-benzyl group in all cases. Instead, Staudinger reduction to give the amine **17** was followed by careful debenzylation with sodium in liquid ammonia,[44] and the crude amino alcohol was then taken up in dioxane/water for Fmoc protection. The resulting alcohol **18** was then subjected to Jones oxidation, as before, to give the protected amino acid **3**.



Scheme 2. Synthesis of Fmoc-Haa9 (**3**). Reagents: a) TrtCl, Et_3N, DCM (100%); b) MsCl, Et_3N, DCM (99%); c) NaH, TBABr, benzyl chloride (99%); d) NaH, DMF (74%) then **9**; e) (i) TFA, Et_3SiH, DCM; (ii) satd. aq. Na_2CO_3 (99%); f) NaH, DMF (75%);g) (i) TFA, Et_3SiH, DCM; (ii) satd. aq. Na_2CO_3 (100%); h) (i) MsCl, Et_3N, DCM; (ii) NaN_3, DMF (85%); i) PPh_3, THF, H_2O (97%); j) (i) Na, NH_3, THF, –78 °C; (ii) Fmoc-Cl, $NaHCO_3$, dioxane, water (88%); k) CrO_3, H_2SO_4, acetone (70%).

We then adapted this approach to give an expedient and high-yielding synthesis of amino acid **2** (Fmoc-Haa7), starting from the tri- and tetraethylene glycol derivatives prepared previously. Thus, tritylated tetraethylene glycol **4** was mesylated and then treated with the sodium salt of benzylated triethylene glycol **10** to give **19** (Scheme 3). This was followed by a similar sequence of detritylation and conversion to the azide **20**, with purification only necessary in the last step. Staudinger reduction of the azide was carried out as previously[33] with purification being effected by filtration and extraction to give **21**. Debenzylation and immediate Fmoc protection again worked successfully to afford **22**, which was converted into the desired amino acid **2** by Jones oxidation, giving an overall yield of 50%. All three synthetic routes could be accomplished on a large scale with few chromatography steps being required.



Scheme 3. Synthesis of Fmoc-Haa7 (**2**). Reagents: a) MsCl, Et_3N, DCM; b) NaH, **10**, DMF; c) TFA, DCM, Et_3SiH; d) satd. aq. Na_2CO_3; e) MsCl, Et_3N, DCM; f) NaN_3, DMF (76% from **4**); g) Ph_3P, THF, H_2O (97%); h) Na, liq. NH_3, –78 °C; i) Fmoc-Cl, $NaHCO_3$, dioxane, water (97%); j) CrO_3, acetone, H_2SO_4 (71%).

Bifunctional Peptides

Lipopolyplex gene delivery vectors, which combine the advantages of polyplexes and lipoplexes, have recently been described by a number of groups. One of the first of these was the receptor-targeted, self-assembling LID vector complex described by Hart et al.[4] This consists of plasmid DNA, a lipid component [a 1:1 mixture of a cationic lipid, DOTMA, and a co-lipid, DOPE (Lipofectin, L)] and a bifunctional, cationic peptide (Peptide **A**) with a K_{16} domain, which binds and condenses DNA, and a cyclic domain (CRRETAWAC), which specifically binds to the $\alpha_5\beta_1$ integrin, allowing the complex to be internalised by receptor-mediated endocytosis. LID vectors have been found to display high transfection efficiency and low toxicity in vitro and in vivo, to transfect non-dividing cells efficiently and were well tolerated with low immunogenicity in vivo.[4,45–47] However, this formulation has not been developed for systemic gene delivery.

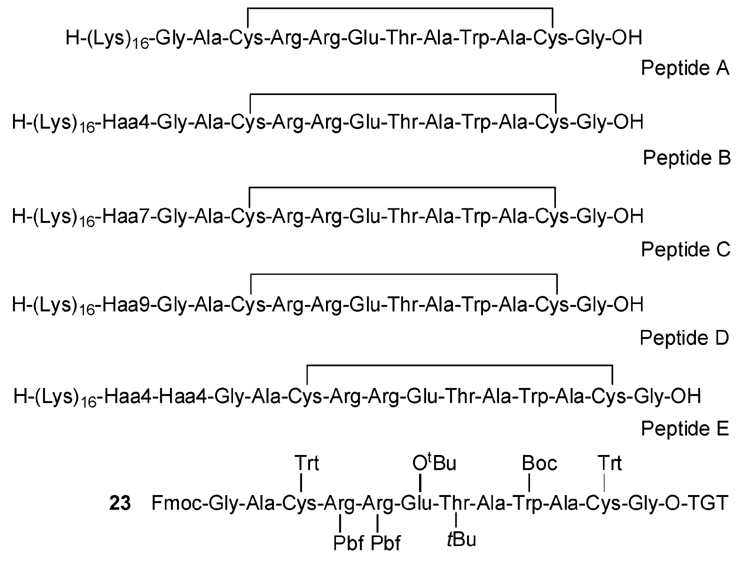


Figure 2. Peptides containing PEG residues, together with the peptide from the original LID vector.

We reasoned that insertion of a PEG spacer between the integrin-binding domain and the K_{16} domain might both stabilise the particle and position the integrin-binding domain further from the complex, making it more accessible to the cell surface. As a first step to designing targeted lipopolyplex vector systems effective for in vivo gene transfer after intravenous administration, we synthesised variants of the bifunctional cationic peptide incorporating PEG-amino acids **1**, **2** and **3**. Thus, peptides **B**, **C** and **D** (Figure 2) bearing the CRRETAWAC integrin-binding domain, PEG-amino acids **1**, **2** and **3**, respectively, and the lysine-rich DNA-binding domain were designed. In order to compare the effects of a single, longer PEG-amino acid with two sequential shorter PEG-amino acids, peptide **E**, containing a repeat of **1** (Haa4) was also synthesised.

As the 12-residue C-terminus is common to all four peptides, the head group **23** was first synthesised on a large scale using the acid-labile NovaSyn-TGT resin preloaded with Fmoc-Gly using standard methods for automated solid-phase synthesis. A portion of the fully protected peptide–resin was then transferred to a Merrifield bubbler. Preliminary experiments to determine the best coupling reagents for the Fmoc-protected PEG-amino acid **2** were then carried out and it was found that the most efficient coupling was effected with TBTU/HOBt/DIPEA. The synthesis of peptide **B** was then completed on the peptide synthesiser using the same reagents to add the remaining lysine residues. Peptides **C**, **D** and **E** were synthesised by automated solid-phase synthesis from the fully protected peptide–resin **23** using TBTU/HOBt/DIPEA with the Fmoc deprotection time and the recycle time both extended by 10 minutes when incorporating amino acids **1** and **3**, respectively. After standard resin cleavage and HPLC purification, the peptides were cyclised by aerial oxidation at high dilution in degassed water over a period of a week. The use of 0.01 M ammonium hydrogen carbonate[48] or DMSO[49] as solvent to speed up the cyclisation was also tried, but residual buffer and solvent interfered with the subsequent HPLC purification, which resulted in low yields of purified peptides.

Transfection of Lipopolyplex Complexes Containing PEG-Linked Peptides

A series of lipopolyplexes **LPD-A**–**E** were formulated from Lipofectin™, plasmid DNA and peptides **A**–**E**, respectively, using the previously described procedure.[4] Additionally, each lipopolyplex was formulated at three different charge ratios: 0.5:1, 1:1 and 4:1.

Transfections of HAEo-, N2A and AJ3.1 cells were then carried out with these lipopolyplexes under different conditions. Figure 3 shows the transfection of HAEo- cells with **LPD-A** (the original LID complex) and **LPD-C** under various conditions. Although poor transfections were obtained with a 0.5:1 ratio for **LPD-C**, with ratios of 4:1, **LPD-C** and **LPD-A** show comparable transfection efficiencies. Figure 3 (b) illustrates that this cell line requires Lipofectin for efficient transfection.

Complexes **LPD-A**, **LPD-C**, **LPD-D** and **LPD-E** were used to transfect HAEo- (Figure 4, a), N2a (Figure 4, b) and AJ3.1 (Figure 4, c) cells. The results were extremely dependent on the cell line. The complex with the peptide with the shortest linker, **LPD-B**, showed good transfection in HAEo- cells but not in the other two cell lines, whereas the complex with the peptide with the longest linker, **LPD-D**, showed good transfection in N2A cells but not in the other two cell lines. **LPD-E** performed poorly in all three cell lines.

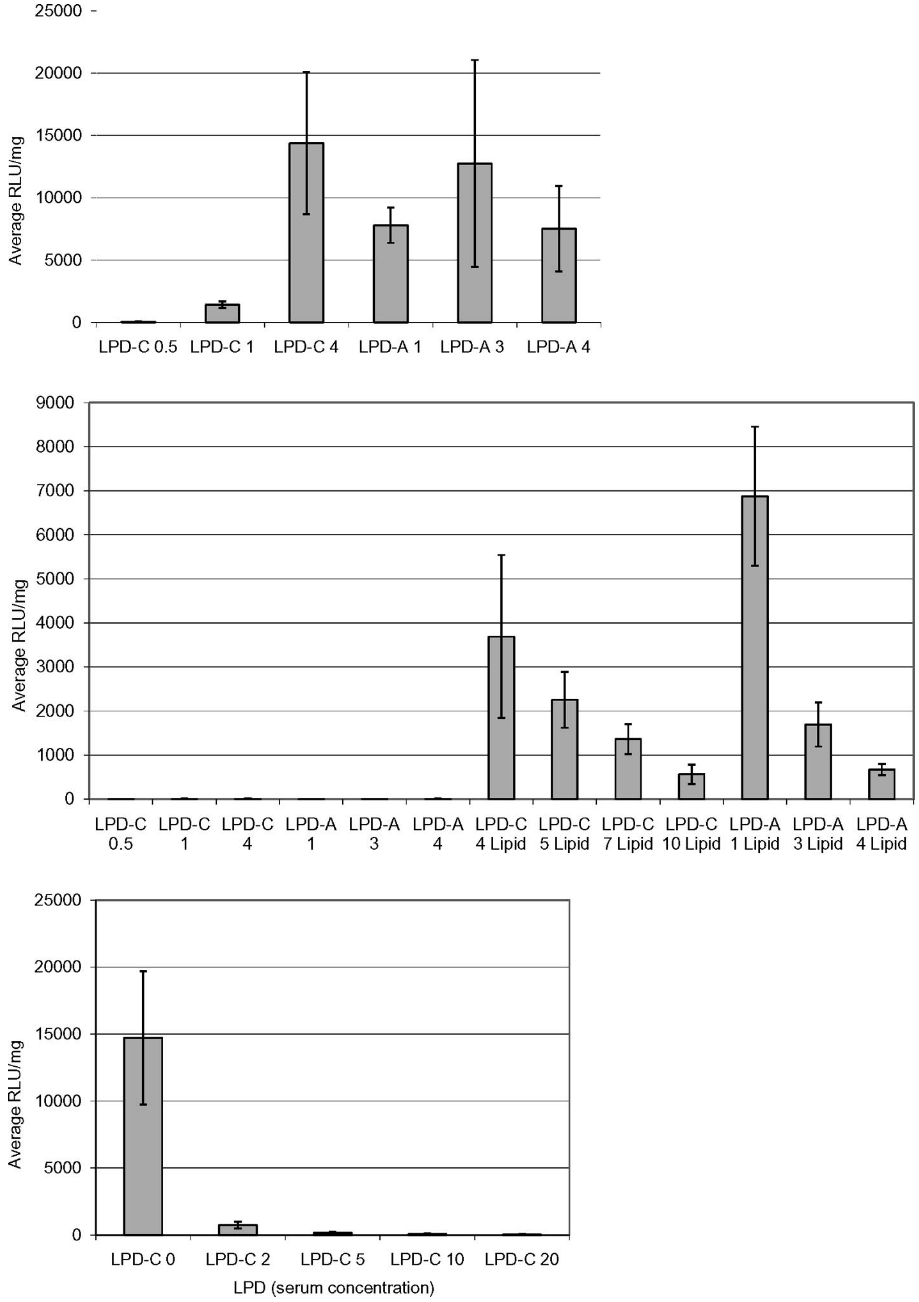


Figure 3. (a) Transfection of HAEo- cells with **LPD-A** and **LPD-C** at different charge ratios, with the DNA/Lipofectin weight ratio constant at 1:0.75. (b) Transfection of HAEo- cells with **LPD-A** and **LPD-C** at different ratios, with and without Lipofectin. (c) Transfection of HAEo- cells with **LPD-C** with increasing amounts of serum and the DNA/Lipofectin weight ratio constant at 1:0.75.

Stability of Lipopolyplex Complex Containing PEG-Linked Peptide C

As a first step towards quantifiying the stability of complexes containing PEG-linked peptides, we investigated the aggregation properties of the most promising lipopolyplex complex, that containing peptide **C**. The lipopolyplex **LPD-C(4:1)** was formulated from Lipofectin, plasmid DNA and peptide **C**, as described previously,[4] and the size of the resulting particles measured at different concentrations of phosphate-buffered saline (PBS) to mimic physiological conditions.

In order to determine the size of the complex, dynamic light scattering (DLS) measurements were taken[50] and the hydrodynamic radius or diameter of the particles calculated.[51,52] The change in size distribution of the lipopolyplex containing peptide **C** over time is shown in Figure 5. It can be clearly seen that, compared with the complex formulated with the original peptide **A** (for which the particle size increases rapidly, indicating extensive aggregation), the PEG-spaced complex is much more stable and aggregates more slowly.

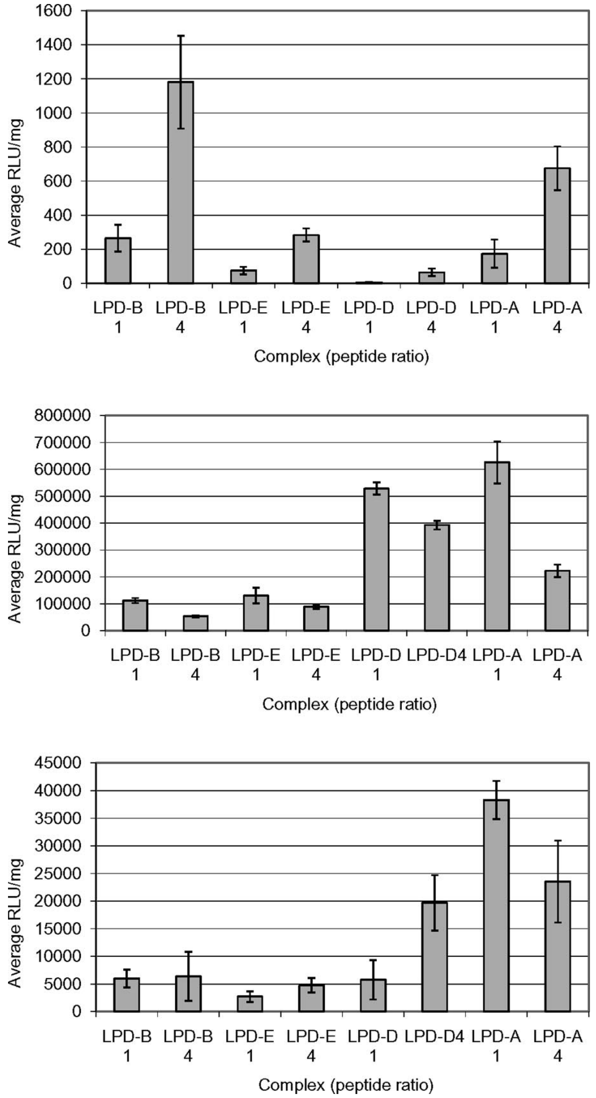


Figure 4. (a) Transfection of HAEo- cells with **LPD-A**, **LPD-B**, **LPD-D** and **LPD-E** at charge ratios of 1:1 and 4:1. (b) Transfection of N2A cells with **LPD-A**, **LPD-B**, **LPD-D** and **LPD-E** at charge ratios of 1:1 and 4:1. (c) Transfection of AJ3.1 cells with **LPD-A**, **LPD-B**, **LPD-D** and **LPD-E** at charge ratios of 1:1 and 4:1. Lipofectin was present throughout at 0.75.

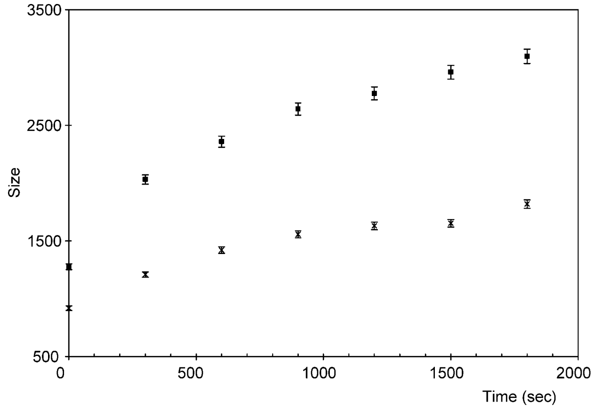


Figure 5. Average aggregate sizes (y axis, nm) in PBS (pH 7.4) as a function of time. Data refer to LID complexes containing peptide A (**LPD-A**) (■) and peptide C (**LPD-C**) (×), respectively.

Conclusions

A series of PEG-amino acids, Fmoc-protected for solid-phase peptide synthesis, have been prepared by a scalable and high-yielding route from cheap and readily available tri- and tetraethylene glycol. The strategy used avoided previously reported problems by first assembling the desired PEG framework, then converting it into the desired Fmoc-protected PEG-amino alcohol and finally oxidising it to the PEG-amino acid in the last step. These amino acids were incorporated into bifunctional peptides bearing integrin-binding and DNA-condensing moieties using standard solid-phase synthesis techniques. Although a range of PEG-spaced amino acids have recently become commercially available, none of them exactly corresponds to the amino acids we have synthesised. We believe that our approach is a valuable synthetic route to PEG-spaced amino acids, which can be adapted to give a variety of linker lengths.

A series of lipopolyplex complexes was then formulated using these PEG-containing bifunctional peptides. The complex **LPD-C**, formulated with a peptide containing Haa7, showed promising transfection results in preliminary experiments. DLS measurements (Figure 5) showed that this lipopolyplex had enhanced stability in PBS compared with a lipopolyplex without a PEG spacer, **LPD-A**. However, this enhanced complex stability does not appear to confer better transfection properties in serum as transfection is greatly reduced at even low serum concentrations (Figure 3, c). Complexes **LPD-B**, formulated with a peptide containing the shorter linker, Haa4, and **LPD-D**, formulated with a peptide containing the longest linker, Haa9, showed variable results, depending on the cell line investigated. In HAEo- cells the complex with the shorter linker, **LPD-B**, showed better transfection than the original vector, **LPD-A**, whereas in N2A cells the complex with the longer linker, **LPD-D**, showed reasonable transfection, although not as good as the original vector, **LPD-A**. In all the cell lines, the complex with two Haa4 amino acids, **LPD-E**, performed poorly.

The reasons for the divergent transfection properties of what appear to be rather similar PEG-containing complexes are not at present clear. In particular, our results indicate that the length of the PEG spacer is not the most important factor, as complexes **LPD-E** and **LPD-C**, which have almost the same length spacer, are so different in transfection efficiency. Clearly in the case of **LPD-E**, the additional amide bond in the centre of the linker must impart additional structural rigidity to the linker, which may be unfavourable for the formation of a stable complex or for its dissociation within the endosome during transfection. A long linker between the integrin-binding and DNA-condensation portions of the peptide does not, however, appear to be necessary for successful transfection. We have recently carried out a detailed biophysical study of the macromolecular structure of the original LID vector, and have demonstrated that the integrin-binding peptide is partly buried in a disordered lipid layer.[53] In light of these recent results, therefore, it seems unlikely that simply increasing the length of the linker between the DNA-condensation moiety and the integrin-binding sequence would have much impact. A more subtle interplay between the different linker structures, the lipid layer and the lipid composition of the different cell lines is clearly operating and will affect the overall stability of the particle and the ease of dissociation of the complex in the endosome. Our preliminary biophysical results indicate that the presence of the PEG spacer in **LPD-C** does impart some degree of additional stability to the complex when suspended in buffer solutions. Further detailed studies of the transfection properties of the PEG-spacer peptides in serum, alone and in combination with PEG-lipids of defined length, are underway and will be reported subsequently.

Experimental Section

General: Solid-phase peptide synthesis was carried out either manually on a Merrifield bubbler or automatically on a peptide synthesiser module (MilliGen 9050Plus PepSynthesiser). All reagents used for either manual or automatic synthesis were purchased from commercial suppliers with the exception of the Fmoc-protected amino acids **1**, **2** and **3**. HIPERSOLV© DMF of HPLC grade was used straight from the bottle, whereas dichloromethane and piperidine were freshly distilled from calcium hydride before use. All peptide syntheses were carried out under nitrogen. Preparative HPLC was carried out on a Waters 600E instrument with a Waters 486 UV detector using a Vydac 218TP reversed-phase HPLC column (25 × 250 mm). Analytical HPLC was carried out on a Varian ProStar 210 with a Varian ProStar 320 UV detector using a Vydac 218TP reversed-phase HPLC column (2.1 × 250 mm). Water/acetonitrile gradients were used as described, with both water and acetonitrile containing 0.1% trifluoroacetic acid. Detection was performed at 215 nm.

11-(Triphenylmethyloxy)-3,6,9-trioxaundecan-1-ol (4): Triphenylmethyl chloride (27.9 g, 100 mmol) in dry dichloromethane (500 mL) was added dropwise to tetraethylene glycol (214 g, 1.10 mol) and triethylamine (20.2 g, 27.8 mL, 200 mmol) in dry dichloromethane (1500 mL). This solution was stirred under argon at room temperature for 24 h. The reaction was then concentrated in vacuo to give a mixture of product and starting material as a yellow oil. This oil was then redissolved in dichloromethane (1000 mL), partitioned with satd. aq. $NaHCO_3$ (500 mL), water (3 × 400 mL) and finally satd. aq. NaCl (500 mL). The organic layer was separated, dried with anhydrous sodium sulfate and concentrated in vacuo to give **4** as a yellow oil (42.2 g, 95%) which was used without further purification. ^1H NMR (300 MHz, CD_3CN): δ = 2.75 (br. s, 1 H, $CH_2O\mathit{H}$), 3.16 [t, J = 5.1 Hz, 2 H, $(C_6H_5)_3COC\mathit{H}_2$], 3.47–3.63 [m, 14 H, $(C\mathit{H}_2OC\mathit{H}_2)_3C\mathit{H}_2OH$], 7.25–7.49 [m, 15 H, $(C_6\mathit{H}_5)_3COCH_2$] ppm. ^{13}C NMR (75 MHz, CD_3CN): δ = 60.8, 63.2, 69.87, 69.91, 70.02, 70.04, 70.2, 72.1, 86.1, 126.8, 127.5, 128.3, 144.0 ppm. IR (oil): $\tilde{\nu}$ = 3440 (O–H), 3057 (aryl-H), 2872 (C–H), 1597 (C=C) cm^{-1}.

1-Amino-11-(triphenylmethyloxy)-3,6,9-trioxaundecane (5): Methanesulfonyl chloride (7.74 mL, 11.45 g, 100 mmol) in dry dichloromethane (50 mL) was added dropwise to 11-(triphenylmethyloxy)-3,6,9-trioxaundecan-1-ol (**4**) (21.82 g, 50 mmol) and triethylamine (15.33 mL, 11.13 g, 110 mmol) in dry dichloromethane (150 mL) under argon at 0 °C. Once addition was complete, the reaction was stirred for 3 h at room temperature. Dichloromethane (200 mL) was then added and the organic solution was partitioned with satd. aq. $NaHCO_3$ (2 × 200 mL). The organic phase was then partitioned with satd. aq. NaCl (200 mL), dried with anhydrous sodium sulfate and concentrated in vacuo to give the mesylate as a brown oil. This oil was dried by azeotropic distillation several times with toluene. The brown oil was dissolved in dimethylformamide (150 mL) and sodium azide (13.0 g, 200 mmol) was added. The reaction was left to stir for 4 d after which time it was concentrated in vacuo and then dissolved in dichloromethane (250 mL). This organic solution was partitioned with water (2 × 150 mL) followed by satd. aq. NaCl (200 mL), dried with anhydrous sodium sulfate and concentrated in vacuo to yield a dark-brown oil. The product was isolated from this oil by normal-phase silica gel chromatography, using a gradient from chloroform/hexane (1:1) to chloroform only, to yield 1-azido-11-(triphenylmethyloxy)-3,6,9-trioxaundecane (21.0 g, 91%) as a yellow oil. ^1H NMR (300 MHz, $CDCl_3$): δ = 3.25 [t, J = 5.1 Hz, 2 H, $(C_6H_5)_3COC\mathit{H}_2$], 3.34 (t, J = 4.8 Hz, 2 H, $C\mathit{H}_2N_3$) 3.63–3.69 [m, 12 H, $(C\mathit{H}_2OC\mathit{H}_2)_3CH_2N_3$], 7.20–7.48 [m, 15 H, $(C_6\mathit{H}_5)_3$] ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 51.1, 63.8, 70.4, 71.1, 71.16, 71.22, 87.0, 127.3, 128.2, 129.2, 144.6 ppm. IR (oil): $\tilde{\nu}$ = 3059 (aryl-H), 2870 (C–H), 2108 (N_3), 1597 (C=C) cm^{-1}. MS (positive ion FAB): m/z (%) = 484 (98) $[M + Na]^+$, 243 (63) $[(C_6H_5)_3C]^+$. HRMS (positive ion FAB): calcd. for $[M + Na]^+$ 484.2212; found 484.2203.

Triphenylphosphane (14.33 g, 54.6 mmol) was added to 1-azido-11-(triphenylmethyloxy)-3,6,9-trioxaundecane (21.0 g, 45.5 mmol) in tetrahydrofuran (100 mL). After 2 h, water (3 mL) was added to the reaction. The reaction was then left to stir under argon at room temperature for 24 h after which time it was concentrated in vacuo. Diethyl ether (65 mL) was added to the remaining oil/solid and the reaction mixture was then filtered and concentrated in vacuo. The product was isolated by normal-phase silica gel chromatography [chloroform, then changing to chloroform/methanol/triethylamine (85:10:5)] to yield **5** (19.0 g, 96%) as a pale-yellow oil. ^1H NMR (300 MHz, $CDCl_3$): δ = 2.83 (t, J = 5.2 Hz, 2 H, $C\mathit{H}_2NH_2$) 3.25 [t, J = 5.2 Hz, 2 H, $(C_6H_5)_3COC\mathit{H}_2$], 3.50 (t, J = 5.2 Hz, 2 H, $C\mathit{H}_2CH_2NH_2$), 3.61–3.69 [m, 10 H, $(C\mathit{H}_2OC\mathit{H}_2)_2C\mathit{H}_2OCH_2$-$CH_2NH_2$], 7.19–7.49 [m, 15 H, $(C_6\mathit{H}_5)_3COCH_2$] ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 42.2, 63.8, 70.8, 71.09, 71.11, 71.14, 71.2, 73.7, 87.0, 127.3, 128.2, 129.2, 144.6 ppm. IR (oil) $\tilde{\nu}$ = 3380 (N–H), 3060 (aryl-H), 2870 (C–H), 1600 (C=C Ar) cm^{-1}. MS (positive ion FAB): m/z (%) = 436 (14) $[M + H]^+$, 243 (99) $[(C_6H_5)_3C]^+$. HRMS (positive ion FAB): calcd. for $[M + H]^+$ 436.2487; found 436.2483.

1-(Fluoren-9-ylmethyloxycarbonylamino)-11-(triphenylmethyloxy)-3,6,9-trioxaundecane (6): Fluoren-9-ylmethyl chloroformate (9.03 g, 35 mmol) in dry dichloromethane (40 mL) was added dropwise to 1-amino-11-(triphenylmethyloxy)-3,6,9-trioxaundecane (**5**) (8.70 g, 20 mmol) and *N*-methylmorpholine (4.4 mL, 4.05 g, 40 mmol) in dry dichloromethane (100 mL) under argon at 0 °C. Once addition was complete, the reaction was stirred at room temperature for 24 h. Dichloromethane (100 mL) was added and the resulting solution partitioned with citric acid solution (pH 6, 150 mL). The organic phase was then partitioned with satd. aq. NaCl (200 mL), dried with anhydrous sodium sulfate and concentrated in vacuo. The product was isolated by normal-phase silica gel chromatography, eluting with chloroform only, to yield **6** as a viscous yellow oil (12.93 g, 95%). ^1H NMR (300 MHz, $CDCl_3$): δ = 3.25 [t, J = 5.2 Hz, 2 H, $(C_6H_5)_3COC\mathit{H}_2$], 3.36 (q, J = 4.5 Hz, 2 H, $C\mathit{H}_2NHFmoc$), 3.55–3.68 [m, 12 H, $(C\mathit{H}_2OC\mathit{H}_2)_3CH_2NHFmoc$], 4.21 (t, J = 6.7 Hz, 1 H, $NHCOOCH_2C\mathit{H}$), 4.40 (d, J = 6.7 Hz, 2 H, $NHCOOC\mathit{H}_2CH$), 5.40 (br. s, 1 H, $N\mathit{H}$), 7.22–7.49 (m, 15 H, aryl Fmoc and trityl), 7.59 (d, J = 7.40 Hz, 2 H, aryl Fmoc), 7.77 (d, J = 7.40 Hz, 2 H, aryl Fmoc) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 41.4, 47.7, 63.8, 67.0, 70.5, 70.8, 71.07, 71.12, 71.2, 87.0, 120.3, 125.5, 127.4, 128.1, 127.4, 128.2, 129.2, 141.7, 144.5, 144.6, 156.9 ppm. IR (oil) $\tilde{\nu}$ = 3350 (N–H), 3060 (aryl-H), 2860 (C–H), 1700 (C=O), 1570–1460 (N–H bend and C=C) cm^{-1}. MS (positive ESI): *m*/*z* (%): 697 (19) [M + K]$^+$, 681 (99) [M + Na]$^+$. HRMS (positive ESI): calcd. for [M + Na]$^+$ 680.29826; found 680.29881.

11-(Fluoren-9-ylmethyloxycarbonylamino)-3,6,9-trioxaundecan-1-ol (7): 1-(Fluoren-9-ylmethyloxycarbonylamino)-11-triphenylmethyloxy-3,6,9-trioxaundecane (**6**) (12.93 g, 19 mmol) was dissolved in a solution of dichloromethane, trifluoroacetic acid and triethylsilane (100 mL, 8:1:1). The reaction was stirred at room temperature under argon for 1 h. Water (100 mL) was then added to the reaction and the resulting mixture was adjusted to pH 7 with satd. aq. $NaHCO_3$ whilst being vigorously stirred. The two layers were separated and the aqueous solution was partitioned with another volume of dichloromethane (100 mL). The organic fractions were combined, dried with anhydrous sodium sulfate and concentrated in vacuo. The product was isolated by normal-phase silica gel chromatography, using a gradient from chloroform (100%) to ethyl acetate (100%), to yield **7** as a yellow oil (7.56 g, 94%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.40 (br. s, 2 H, $\mathrm{C}H_2\mathrm{NHFmoc}$), 3.57–3.74 [m, 14 H, $\mathrm{HOC}H_2(\mathrm{C}H_2\mathrm{OC}H_2)_3$], 4.21 (t, *J* = 6.6 Hz, 1 H, $\mathrm{NHCOOCH_2C}H$), 4.42 (d, *J* = 6.6 Hz, 2 H, $\mathrm{NHCOOC}H_2\mathrm{CH}$), 4.76, 6.06 (br. s, 2 H, N*H* and O*H*), 7.30 (t, *J* = 7.4 Hz, 2 H), 7.38 (t, *J* = 7.4 Hz, 2 H), 7.61 (d, *J* = 7.4 Hz, 2 H, aryl Fmoc), 7.76 (d, *J* = 7.4 Hz, 2 H, aryl Fmoc) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 41.4, 47.8, 61.7, 67.1, 70.1, 70.4, 71.5, 71.3, 72.5, 120.2, 125.4, 127.4, 127.9, 141.6, 144.3, 157.6 ppm. IR (oil) $\tilde{\nu}$ = 3324 (N–H and O–H), 3067, 3041, 3020 (aryl-H), 2882 (C–H), 1672 (C=O), 1535 (N–H) cm^{-1}. MS (positive ion FAB): *m*/*z* (%): 454 (92) [M + K]$^+$, 438 (99) [M + Na]$^+$, 179 (34) $[C_{14}H_{11}]^+$. HRMS (positive ion FAB): calcd. for [M + Na]$^+$ 438.1893; found 438.1886.

11-(Fluoren-9-ylmethyloxycarbonylamino)-3,6,9-trioxaundecanoic Acid (Fmoc-Haa4, 1):[29] Chromium trioxide (3.00 g, 30 mmol) in sulfuric acid (1.5 M, 60.0 mL, 90 mmol) was very slowly added to vigorously stirring 11-(fluoren-9-ylmethyloxycarbonylamino)-3,6,9-trioxaundecan-1-ol (**7**) (4.15 g, 10 mmol) in acetone (100 mL) at 0 °C. Once addition was complete the reaction was left to warm to room temperature and to stir for 24 h. Water (300 mL) was then added to the reaction along with reversed-phase silica gel (100 g). The reaction mixture was concentrated in vacuo until the volume had decreased by approximately one quarter and more water (100 mL) was added. The reaction mixture was once again concentrated in vacuo until no acetone could be detected in the mixture. The mixture was then filtered to recover the reversed-phase silica gel, which was washed with copious amounts of water until the silica gel was almost colourless. The reversed-phase silica gel was then washed with acetonitrile (4 × 200 mL) and chloroform (3 × 150 mL). The organic fractions were combined and concentrated in vacuo to give a pale-green oil. The product was isolated by normal-phase silica gel chromatography, eluting with a gradient from chloroform (100%) to chloroform/methanol (95:5) and then to chloroform/methanol/acetic acid (80:5:5), to yield **1** as a yellow oil (2.93 g, 68%) spectroscopically identical to the literature.[29] ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.39 (m, 2 H, $\mathrm{C}H_2\mathrm{NHFmoc}$), 3.54–3.71 [m, 10 H, $\mathrm{OC}H_2(\mathrm{C}H_2\mathrm{OC}H_2)_2\mathrm{CH_2NHFmoc}$], 4.12 (s, 2 H, $\mathrm{HO_2CC}H_2$), 4.21 (t, *J* = 6.7 Hz, 1 H, $\mathrm{NHCOOCH_2C}H$), 4.39 (d, *J* = 6.7 Hz, 2 H, $\mathrm{NHCOOC}H_2\mathrm{CH}$), 5.70 (br. s, 1 H, N*H*), 7.30 (t, *J* = 7.4 Hz, 2 H), 7.38 (t, *J* = 7.4 Hz, 2 H), 7.59 (d, *J* = 7.4 Hz, 2 H), 7.74 (d, *J* = 7.4 Hz, 2 H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 41.3, 47.7, 67.1, 69.1, 70.4, 70.6, 70.8, 71.5, 120.3, 125.5, 127.5, 128.1, 141.7, 144.4, 157.3, 173.1 ppm. IR (oil) $\tilde{\nu}$ = 3336 (COO–H), 3060 (aryl-H), 2880 (C–H), 1715, 1700 (C=O), 1611 (N–H bend) cm^{-1}. MS (positive ES): *m*/*z* (%) = 452 (99) [M + Na]$^+$. HRMS (positive ESI): calcd. for [M + Na]$^+$ 452.16797; found 452.16899.

8-(Triphenylmethyloxy)-3,6-dioxaoctan-1-ol (8):[54] Triphenylmethyl chloride (6.07 g, 30 mmol) in dry dichloromethane (150 mL) was added dropwise to triethylene glycol (90.1 g, 600 mmol) and triethylamine (6.07 g, 8.36 mL, 60 mmol) in dry dichloromethane (500 mL). This solution was stirred under nitrogen at room temperature for 24 h. The reaction was then concentrated in vacuo to give a mixture of product and starting material as a yellow oil. This oil was redissolved in dichloromethane (400 mL), partitioned with satd. aq. $NaHCO_3$ (200 mL), water (4 × 250 mL) and finally satd. aq. NaCl (250 mL). The organic layer was separated, dried with anhydrous sodium sulfate and concentrated in vacuo to give a yellow oil. This oil was purified by normal-phase silica gel chromatography, eluting with a gradient from chloroform (100%) to chloroform/methanol (98:2), to yield **8** as a pale-yellow oil (12.0 g, 100%) spectroscopically identical to the literature.[54] ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.35 (br. s, 1 H, $\mathrm{CH_2O}H$), 3.25 [t, *J* = 5.1 Hz, 2 H, $(\mathrm{C_6H_5})_3\mathrm{COC}H_2$], 3.58–3.71 [m, 10 H, $(\mathrm{C}H_2\mathrm{OC}H_2)_2\mathrm{C}H_2\mathrm{OH}$], 7.18–7.47 [m, 15 H, $(\mathrm{C_6}H_5)_3\mathrm{COCH_2}$] ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 62.2, 63.7, 71.0, 71.1, 71.2, 73.0, 87.1, 127.4, 128.2, 129.1, 144.5 ppm. IR (oil): $\tilde{\nu}$ = 3440 (O–H), 3057 (aryl-H), 2872 (C–H), 1597 (C=C) cm^{-1}. MS (positive ion FAB): *m*/*z* (%) = 415 (25) [M + Na]$^+$. HRMS (positive ion FAB): calcd. for [M + Na]$^+$ 415.1885; found 415.1876.

8-(Triphenylmethyloxy)-3,6-dioxaoctyl Methanesulfonate (9):[55] Methanesulfonyl chloride (5.80 mL, 8.59 g, 75 mmol) in dry dichloromethane (40 mL) was slowly added to 8-(triphenylmethyloxy)-3,6-dioxaoctan-1-ol (**8**) (12.0 g, 30 mmol) and triethylamine (9.1 g, 12.5 mL, 90 mmol) in dry dichloromethane (100 mL) under nitrogen at 0 °C. Once addition was complete, the reaction was left to warm to room temperature. After 2.5 h, excess satd. aq. $NaHCO_3$ was added and the reaction was stirred for 10 min. Additional dichloromethane (200 mL) was added, the aqueous and organic layers were separated and the organic solution was partitioned with satd. aq. NaCl (100 mL). The aqueous solution was back-extracted with dichloromethane (150 mL), the organic solutions were combined, dried with anhydrous sodium sulfate and concentrated in vacuo to give **9** as an orange oil. The oil was then azeotroped several times with dry toluene. This oil became a red solid (14.1 g, 99.8%) after several hours in high vacuo. The product, identical by NMR to the literature,[55] was used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.94 (s, 3 H, $\mathrm{C}H_3\mathrm{SO_3CH_2}$), 3.25 [t, *J* = 5.1 Hz, 2 H, $\mathrm{C}H_2\mathrm{OC(C_6H_5)_3}$], 3.64–3.76 [m, 6 H, $\mathrm{CH_3SO_3CH_2CH_2O(C}H_2)_2\mathrm{OC}H_2$], 3.78 (m, 2 H, $\mathrm{CH_3SO_3CH_2}$-$\mathrm{C}H_2\mathrm{O}$), 4.35 (m, 2 H, $\mathrm{CH_3SO_3C}H_2\mathrm{CH_2O}$), 7.20–7.48 [m, 15 H, $\mathrm{OC(C_6}H_5)_3$] ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 38.0, 63.8, 69.5, 69.7, 71.15, 71.2, 87.0, 127.4, 128.2, 129.1, 144.5 ppm.

8-(Phenylmethyloxy)-3,6-dioxaoctan-1-ol (10):[56,57] Sodium hydride (1.20 g, 60% w/w in mineral oil, 30 mmol) was added cautiously to triethylene glycol (30.0 g, 200 mmol) and tetrabutylammonium bromide (0.5 g, cat.) in dry tetrahydrofuran (150 mL). After stirring for 20 min, benzyl chloride (2.53 g, 20 mmol) was added and the reaction was heated at reflux under nitrogen for 24 h. The reaction was then cooled to room temperature and concentrated in vacuo to give an oil. This oil was dissolved in dichloromethane (300 mL) and partitioned with satd. aq. NaCl (2 × 200 mL). The organic phase was dried with anhydrous sodium sulfate and concentrated in vacuo to give a yellow oil. This was purified by normal-phase silica gel chromatography, eluting with a gradient from chloroform (100%) to chloroform/methanol (90:10), to yield **10** as a pale-yellow oil (4.94 g, 99%) spectroscopically identical to the literature.[56,57] ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.74 (br. t, 1 H, CH_2OH), 3.60–3.70 [m, 12 H, $OCH_2(CH_2OCH_2)_2CH_2OH$], 4.57 (s, 2 H, $C_6H_5CH_2O$), 7.25–7.35 (m, 5 H, $C_6H_5CH_2O$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 62.1, 69.8, 70.8, 71.0, 71.1, 72.9, 73.7, 128.0, 128.1, 128.7, 138.1 ppm. IR (oil): $\tilde{\nu}$ = 3449 (O–H), 3030 (aryl-H), 2868 (C–H) cm^{-1}. MS (positive ion FAB): *m/z* (%) = 263 (4) [M + Na]$^+$, 241 (99) [M + H]$^+$, 91 (100) [C_7H_7]$^+$. HRMS (positive ion FAB): calcd. for [M + H]$^+$ 241.1440; found 241.1442.

1-(Triphenylmethyloxy)-17-(phenylmethyloxy)-3,6,9,12,15-pentaoxaheptadecane (11): Sodium hydride (2.4 g, 60% w/w in mineral oil, 60 mmol) was added to 8-(phenylmethyloxy)-3,6-dioxaoctan-1-ol (**10**) (4.94 g, 20 mmol) and 8-(triphenylmethyloxy)-3,6-dioxaoctyl methanesulfonate (**9**) (9.88 g, 21 mmol) in dry dimethylformamide (50 mL) under argon. This solution was stirred for 5 d at room temperature. The reaction was then concentrated in vacuo and then redissolved in diethyl ether (200 mL) and water (200 mL). The organic phase was then partitioned with aq. satd. NaCl with a little $NaHCO_3$ (200 mL), dried with anhydrous sodium sulfate and concentrated in vacuo to give a viscous dark-brown oil. This oil was purified by normal-phase silica gel chromatography, eluting with a gradient from chloroform (100%) to chloroform/methanol (98:2), to yield **11** as an orange oil (9.11 g, 74.1%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.25 [t, *J* = 5.2 Hz, 2 H, $CH_2OC(C_6H_5)_3$], 3.64–3.69 [m, 22 H, $OCH_2(CH_2OCH_2)_5$], 4.57 (s, 2 H, $C_6H_5CH_2OCH_2$), 7.20–7.52 [m, 20 H, $C_6H_5CH_2OCH_2(CH_2OCH_2)_5CH_2OC(C_6H_5)_3$] ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 63.8, 69.9, 71.03, 71.07, 71.10, 71.2, 73.7, 87.0, 127.3, 128.0, 128.1, 128.2, 128.7, 129.2, 138.8, 144.6 ppm. IR (oil): $\tilde{\nu}$ = 3030 (aryl-H), 2869 (C–H), 1600, 1570 (C=C) cm^{-1}. MS (positive ion FAB): *m/z* (%) = 637 (0.6) [M + Na]$^+$, 613 (1) [$C_{38}H_{45}O_7$]$^+$, 243 (100) [$C_{19}H_{15}$]$^+$. HRMS (positive ion FAB, NOBA matrix): calcd. for [M + Na]$^+$ 637.3141; found 637.3148.

17-(Phenylmethyloxy)-3,6,9,12,15-pentaoxaheptadecan-1-ol (12):[57,58] A solution made up of dichloromethane, triethylsilane and trifluoroacetic acid (100 mL, 80:10:10) was added to 17-(phenylmethyloxy)-1-(triphenylmethyloxy)-3,6,9,12,15-pentaoxaheptadecane (**11**) (9.11 g, 14.82 mmol). The reaction was stirred at room temperature for 1 h. Satd. aq. Na_2CO_3 was then slowly added to the reaction with vigorous stirring until the reaction reached pH 12. The two layers were partitioned and the aqueous fraction was back-extracted several times with dichloromethane (6 × 100 mL). The organic fractions were combined, dried with anhydrous sodium sulfate and concentrated in vacuo to give a yellow solid/oil. This oil was redissolved in water (100 mL) and the aqueous solution was partitioned twice with hexane (2 × 100 mL). The aqueous solution was then concentrated in vacuo to give a yellow oil. The resulting oil was dried by azeotropic distillation with anhydrous acetonitrile until **12** was recovered (5.51g, 99%) as an oil, spectroscopically identical to the data given in the literature,[57,58] which required no further purification. ^{1}H NMR (300 MHz, CD_2Cl_2): δ = 3.62–3.79 [m, 22 H, $(CH_2OCH_2)_5CH_2OH$], 4.60 (s, 2 H, $C_6H_5CH_2O$), 7.29–7.38 (m, 5 H, $C_6H_5CH_2O$) ppm. ^{13}C NMR (75 MHz, CD_2Cl_2): δ = 60.9, 69.1, 69.79, 69.82, 69.9, 70.10, 70.14, 70.2, 70.5, 72.1, 73.9, 127.6, 127.7, 128.3, 138.1 (two signals superimposed) ppm. IR (oil): $\tilde{\nu}$ = 3474 (O–H), 3030 (aryl-H), 2868 (C–H) cm^{-1}. MS (positive ion FAB): *m/z* (%) = 395 (5) [M + Na]$^+$, 373 (16) [$C_{19}H_{33}O_7$]$^+$. HRMS (positive ion FAB, NOBA matrix): calcd. for [M + Na]$^+$ 395.2046; found 395.2051.

1-(Triphenylmethyloxy)-26-(phenylmethyloxy)-3,6,9,12,15,18,21,24-octaoxahexaeicosane (14):[43b] 8-(Triphenylmethyloxy)-3,6-dioxaoctyl methanesulfonate (**9**) (28.23 g, 60 mmol) and sodium hydride (4.00 g, 60% w/w in mineral oil, 100 mmol) was added to 17-(phenylmethyloxy)-3,6,9,12,15-pentaoxaheptadecan-1-ol (**12**) (16.38 g, 43.9 mmol) in dry dimethylformamide (250 mL). The reaction was left to stir for 4 d at room temperature after which time it was concentrated in vacuo. The reaction mixture was redissolved in diethyl ether (400 mL) and cooled in an ice bath to 0 °C. Water was then slowly added until hydrogen was no longer evolved. The organic phase was then partitioned twice with aq. satd. NaCl with a little $NaHCO_3$ (2 × 300 mL), dried with anhydrous sodium sulfate and concentrated in vacuo to give a viscous dark-brown oil. The product was isolated by normal-phase silica gel chromatography, eluting with ethyl acetate only to yield **14** as a yellow oil (24.2 g, 75.0%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.24 [t, *J* = 5.2 Hz, 2 H, $CH_2OC(C_6H_5)_3$], 3.57–3.69 [m, 34 H, $C_6H_5CH_2OCH_2(CH_2OCH_2)_8$], 4.56 (s, 2 H, $C_6H_5CH_2OCH_2$), 7.21–7.48 [m, 20 H, $C_6H_5CH_2OCH_2(CH_2OCH_2)_8CH_2OC(C_6H_5)_3$] ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 63.4, 69.5, 70.6, 70.7, 70.8, 73.2, 86.6, 126.9, 127.6, 127.7, 128.4, 128.7, 138.4, 144.2 (two signals superimposed) ppm. IR (oil): $\tilde{\nu}$ = 3058, 3031 (aryl-H), 2868 (C–H), 1962 (C=C), 1596 (C=C stretch) cm^{-1}. MS (positive ion FAB): *m/z* (%) = 769 (75) [M + Na]$^+$, 243 (39) [$(C_6H_5)_3C$]$^+$. HRMS (positive ion FAB): calcd. for [M + Na]$^+$ 769.39222; found 769.39298.

26-(Phenylmethyloxy)-3,6,9,12,15,18,21,24-octaoxahexaeicosan-1-ol (15):[58] A solution of trifluoroacetic acid (20 mL) and triethylsilane (20 mL) in dichloromethane (160 mL) was added to 1-(triphenylmethyloxy)-26-(phenylmethyloxy)-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**14**) (24.0 g, 32.1 mmol). The reaction was left to stir at room temperature for 1 h, after which satd. aq. Na_2CO_3 was slowly added to the reaction with vigorous stirring until the reaction reached pH 12. The two layers were partitioned and the aqueous fraction was back-extracted several times with dichloromethane (9 × 100 mL). The organic fractions were combined, dried with anhydrous sodium sulfate and concentrated in vacuo to give a yellow solid/oil. This oil was redissolved in water (200 mL) and the aqueous solution was partitioned twice with hexane (2 × 200 mL). The aqueous solution was then concentrated in vacuo to give a yellow oil. The resulting opaque oil was dried by azeotropic distillation with anhydrous acetonitrile until **15** was obtained as a colourless oil (16.2 g, 99.9%), spectroscopically identical to the literature data.[58] ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.57–3.73 [m, 36 H, $C_6H_5CH_2OCH_2(CH_2OCH_2)_8CH_2OH$], 4.55 (s, 2 H, $C_6H_5CH_2O$), 7.21–7.33 (m, 5 H, $C_6H_5CH_2O$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 61.6, 69.5, 70.2, 70.4, 70.5, 70.55, 70.62, 72.4, 73.2, 127.6, 127.7, 128.3, 138.3 (two signals superimposed) ppm. IR (oil): $\tilde{\nu}$ = 3333 (O–H), 3030 (aryl-H), 2869 (C–H), 1455 (C=C) cm^{-1}. MS (positive ESI): *m/z* (%) = 543 (3) [M + K]$^+$, 527 (99) [M + Na]$^+$. HRMS (positive ESI): calcd. for [M + Na]$^+$ 527.2826; found 527.2850.

1-Azido-26-(phenylmethyloxy)-3,6,9,12,15,18,21,24-octaoxahexaeicosane (16): Methanesulfonyl chloride (7.43 mL, 11.0 g, 96 mmol) in dry dichloromethane (75 mL) was slowly added to 26-(phenylmethyloxy)-3,6,9,12,15,18,21,24-octaoxahexaeicosan-1-ol (**15**) (16.2 g, 32.1 mmol) and triethylamine (11.3 g, 15.60 mL, 112 mmol) in dry dichloromethane (200 mL) under nitrogen at 0 °C. Once addition was complete, the reaction was left to warm to room temperature. After 4 h, excess satd. aq. $NaHCO_3$ was added and the reaction was stirred for 10 min. Additional dichloromethane (300 mL) was added, the aqueous and organic layers were separated and the organic solution was partitioned with satd. aq. NaCl (250 mL). The aqueous solution was back-extracted with dichloromethane (2 × 250 mL), the organic solutions were combined, dried with anhydrous sodium sulfate and concentrated in vacuo to give an orange oil. The oil was then azeotroped several times with dry toluene. This oil became a red solid after several hours under high vacuo. This red solid was redissolved in dry dimethylformamide (200 mL) and sodium azide (10.4 g, 160 mmol) was added. The reaction was stirred for 72 h at room temperature under argon, after which the reaction was concentrated in vacuo. Xylene (250 mL) was then added and the reaction was once again concentrated in vacuo. The reaction mixture was triturated with diethyl ether (750 mL) and filtered, the filtrate being concentrated in vacuo to give an orange oil. The product was isolated by normal-phase silica gel chromatography, eluting with a gradient from ethyl acetate (100%) to ethyl acetate/methanol (95:5), to yield **16** as a yellow oil (14.4 g, 85%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.36 (t, *J* = 5.1 Hz, 2 H, CH_2N_3), 3.58–3.70 [m, 34 H, $CH_2(CH_2OCH_2)_8$-CH_2N_3], 4.55 (s, 2 H, $C_6H_5CH_2OCH_2$), 7.21–7.33 (m, 5 H, $C_6H_5CH_2O$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 50.7, 69.5, 70.0, 70.6, 70.7, 73.2, 127.5, 127.7, 128.3, 138.3 (signals superimposed) ppm. IR (oil): $\tilde{\nu}$ = 3020 (aryl-H), 2866 (C–H), 2106 (N_3), 1454 (C=C) cm^{-1}. MS (positive ES): *m/z* (%) = 568 (2) $[M + K]^+$, 552 (99) $[M + Na]^+$, 524 (3) $[C_{25}H_{43}O_9N + Na]^+$. HRMS (positive ion FAB): calcd. for $[M + Na]^+$ 552.2897; found 552.2906.

1-Amino-26-(phenylmethyloxy)-3,6,9,12,15,18,21,24-octaoxahexaeicosane (17): Triphenylphosphane (2.89 g, 11 mmol) was added to 1-azido-26-(phenylmethyloxy)-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**16**) (5.29 g, 10 mmol) in tetrahydrofuran (50 mL). The reaction was stirred for 2 h at room temperature and water (0.8 mL, 44 mmol) was then added. The reaction was then stirred for 48 h at room temperature. The reaction mixture was then concentrated in vacuo, redissolved in toluene (100 mL) and then concentrated in vacuo once more. The product was isolated from the resulting semi-solid by normal-phase silica gel chromatography, eluting with a gradient from chloroform (100%) to chloroform/methanol/triethylamine (90:5:5), to afford **17** as a pale-yellow oil (4.86 g, 97%). ^{1}H NMR (300 MHz, DMSO): δ = 2.67 (t, *J* = 5.8 Hz, 2 H, CH_2NH_2), 3.38 (t, *J* = 5.8 Hz, 2 H, $CH_2CH_2NH_2$), 3.49–3.61 [m, 32 H, $C_6H_5CH_2O(CH_2CH_2O)_8$], 4.51 (s, 2 H, $C_6H_5CH_2O$), 7.27–7.39 (m, 5 H, $C_6H_5CH_2O$) ppm. ^{13}C NMR (75 MHz, DMSO): δ = 42.2, 70.1, 70.5, 70.7, 72.9, 73.8, 128.2, 128.4, 129.1, 139.4 (signals superimposed) ppm. IR (oil): $\tilde{\nu}$ = 3269 (N–H), 3050 (aryl-H), 2869 (C–H), 1601 (N–H bend), 1452 (C=C) cm^{-1}. MS (positive ES): *m/z* (%) = 526 (8) $[M + Na]^+$, 504 (99) $[M + H]^+$, 524 (13) $[C_{18}H_{40}O_9N]^+$. HRMS (positive ESI): calcd. for $[M + H]^+$ 504.31671; found 504.31645.

26-(Fluoren-9-ylmethyloxycarbonylamino)-3,6,9,12,15,18,21,24-octaoxahexaeicosan-1-ol (18): A solution of 1-amino-26-(phenylmethyloxy)-3,6,9,12,15,18,21,24-octaoxahexaeicosane (**17**) (5.51 g, 10.9 mmol) in dry diethyl ether (50 mL) under argon was cooled to –78 °C. Ammonia was condensed into this solution until the volume of the solution had almost doubled (ca. 100 mL). Sodium pellets were added to the solution under argon at –78 °C until a dark-blue colour persisted. The reaction was warmed from –78 to –30 °C and then cooled back to –78 °C. Methanol was slowly added to the reaction at –78 °C under argon until the solution was no longer blue in colour. The reaction was then warmed to room temperature and the majority of the ammonia was allowed to evaporate. The reaction was concentrated in vacuo and then kept under high vacuum for 24 h to ensure that as little ammonia as possible was present in the remaining residue. Water (100 mL) was then added to the remaining residue and the pH was adjusted to 4 (concd. hydrochloric acid). The pH of the solution was then adjusted to 7 by addition of solid $NaHCO_3$. Once the solution had been neutralised, $NaHCO_3$ (1.38 g, 16.41 mmol) was again added, followed by water (50 mL) and dioxane (100 mL). 9-Fluorenylmethyl chloroformate (4.25 g, 16.41 mmol) in dioxane (50 mL) was slowly added to this solution, cooled to 0 °C. The reaction was then warmed to room temperature and left to stir for 6 h. After 6 h the solution was acidified to pH 6 by addition of solid citric acid and then concentrated in vacuo to remove dioxane. The remaining aqueous solution was partitioned three times with chloroform (3 × 250 mL). The organic fractions were combined, dried with anhydrous sodium sulfate and concentrated in vacuo to give a yellow oil. This oil was purified by normal-phase silica gel chromatography, eluting with ethyl acetate/methanol (4:1), to yield **18** as a yellow oil (6.10 g, 88%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.71 (br. s, 1 H, OH), 3.39 (m, 2 H, $CH_2NHFmoc$), 3.55–3.73 [m, 34 H, $HOCH_2(CH_2OCH_2)_8$], 4.22 (t, *J* = 6.8 Hz, 1 H, $NHCOOCH_2CHC_{12}H_8$), 4.41 (d, *J* = 6.8 Hz, 2 H, $CH_2NHCOCH_2$), 5.50 (br. s, 1 H, NH), 7.31 (t, *J* = 7.4 Hz, 2 H, aryl Fmoc), 7.39 (t, *J* = 7.4 Hz, 2 H, aryl Fmoc), 7.61 (d, *J* = 7.4 Hz, 2 H, aryl Fmoc), 7.76 (d, *J* = 7.4 Hz, 2 H, aryl Fmoc) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 41.0, 47.3, 61.7, 66.6, 70.1, 70.4, 70.6, 72.6, 119.9, 125.1, 127.0, 127.7, 141.3, 144.0, 156.6 ppm. IR (oil): $\tilde{\nu}$ = 3325 (O–H and N–H), 3066, 3041, 3020 (aryl-H), 2883 (C–H), 1672 (C=O stretch and N–H bend), 1452 (C=C) cm^{-1}. MS (positive ion FAB): *m/z* (%) = 658 (8) $[M + Na]^+$, 636 (32) $[M + H]^+$, 414 (21) $[C_{18}H_{40}O_9N]^+$, 179 (68) $[C_{14}H_{11}]^+$. HRMS (positive ion FAB): calcd. for $[M + H]^+$ 636.3384; found 636.3380.

26-(Fluoren-9-ylmethyloxycarbonylamino)-3,6,9,12,15,18,21,24-octaoxahexaeicosanoic Acid (Fmoc-Haa9, 3): Chromium trioxide (2.88 g, 28.8 mmol) in sulfuric acid (1.5 M, 57.6 mL, 86.4 mmol) was very slowly added to vigorously stirring 26-(fluoren-9-ylmethyloxycarbonylamino)-3,6,9,12,15,18,21,24-octaoxahexaeicosan-1-ol (**18**) (6.10 g, 9.60 mmol) in acetone (125 mL) at 0 °C. Once addition was complete the reaction was left to warm to room temperature and stirred for 24 h. Water (400 mL) was then added to the reaction along with reversed-phase silica gel (120 g). The reaction mixture was concentrated in vacuo until the volume had decreased by approximately one quarter and more water (100 mL) was added. The reaction mixture was once again concentrated in vacuo until no acetone could be detected in the mixture. The mixture was then filtered to recover the reversed-phase silica gel which was washed with copious amounts of water until the silica gel was almost colourless. The reversed-phase silica gel was then washed with acetonitrile (4 × 200 mL) and chloroform (3 × 150 mL). The organic fractions were combined and concentrated in vacuo to give a pale-green oil. The product was isolated by normal-phase silica gel chromatography, eluting on a gradient from chloroform to chloroform/methanol (95:5) and finally to chloroform/methanol/acetic acid (80:15:5), to yield **3** as a yellow oil (4.38 g, 70.0%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.38 (m, 2 H, $CH_2NHCOCH_2C_{13}H_9$), 3.54–3.73 [m, 30 H, $HO_2CCH_2OCH_2(CH_2OCH_2)_7$], 4.14 [s, 2 H, $HO_2CCH_2(OCH_2CH_2)_7$], 4.21 (t, *J* = 6.9 Hz, 1 H, $NHCOOCH_2CHC_{12}H_8$), 4.39 (d, *J* = 6.9 Hz, 2 H, $CH_2NHCOCH_2C_{13}H_9$), 5.54 (br. s, 1 H, NH), 7.31 (t, *J* = 7.4 Hz, 2 H, aryl Fmoc), 7.38 (t, *J* = 7.4 Hz, 2 H, aryl Fmoc), 7.59 (d, *J* = 7.4 Hz, 2 H, aryl Fmoc), 7.74 (d, *J* = 7.4 Hz, 2 H, aryl Fmoc) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 40.9, 47.3, 66.6, 70.1, 70.3, 70.5, 70.6, 71.1, 119.9, 125.1, 127.1, 127.7, 141.3, 144.0, 156.7, 171.6 ppm. IR (oil): $\tilde{\nu}$ = 3336 (COO–H), 3060 (aryl-H), 2881, 1714 (C=O), 1700 (C=O), 1600 (C=C and N–H bend) cm^{-1}. MS (positive ES): *m/z* (%) = 672 (2) $[M + Na]^+$, 650 (99) $[M + H]^+$, 428 (4) $[C_{18}H_{38}O_{10}N]^+$. HRMS (positive ESI): calcd. for $[M + Na]^+$ 672.29905; found 672.299822.

1-Azido-20-(phenylmethyloxy)-3,6,9,12,15,18-hexaoxaeicosane (20): Methanesulfonyl chloride (15.48 mL, 22.91 g, 200 mmol) in dry dichloromethane (100 mL) was added dropwise to 11-(triphenylmethyloxy)-3,6,9-trioxaundecan-1-ol (**4**) (43.65 g, 100 mmol) and triethylamine (30.66 mL, 22.26 g, 220 mmol) in dry dichloromethane (250 mL) under argon at 0 °C. Once addition was complete, the reaction was stirred for 5 h at room temperature. A further volume of dichloromethane (250 mL) was then added to the reaction and the organic solution was partitioned with satd. aq. $NaHCO_3$ (2 × 300 mL). The organic phase was then partitioned with satd. aq. NaCl (400 mL), dried with anhydrous sodium sulfate and concentrated in vacuo to give a brown oil. This oil was dried by azeotropic distillation several times with toluene and then placed under high vacuum until the oil became an orange solid. 8-(Phenylmethyloxy)-3,6-dioxaoctan-1-ol (**10**) (28.34 g, 100 mmol) in dry dimethylformamide (200 mL) was added to the orange solid under argon followed by sodium hydride (12.0 g, 300 mmol, 60% w/w in mineral oil). The reaction was stirred for 5 d at room temperature. The reaction was then concentrated in vacuo and then redissolved in diethyl ether (1000 mL) and water (500 mL). The organic phase was then partitioned with aq. satd. NaCl with a little $NaHCO_3$ (500 mL), dried with anhydrous sodium sulfate and concentrated in vacuo to give **19** as a brown oil. A solution made up of dichloromethane, triethylsilane and trifluoroacetic acid (300 mL, 80:10:10) was added to this brown oil. The reaction was left to stir at room temperature for 2 h. After 2 h, satd. aq. Na_2CO_3 was slowly added to the reaction with vigorous stirring until the reaction reached pH 12. The two layers were partitioned and the aqueous fraction was back-extracted several times with dichloromethane (9 × 100 mL). The organic fractions were combined, dried with anhydrous sodium sulfate and concentrated in vacuo to give a yellow solid/oil. This oil was redissolved in water (200 mL) and the aqueous solution was partitioned twice with hexane (2 × 200 mL). The aqueous solution was then concentrated in vacuo to give a pale-yellow oil. The resulting oil was dried by azeotropic distillation with anhydrous acetonitrile several times. Triethylamine (30.66 mL, 22.26 g, 220 mmol) in dry dichloromethane (250 mL) was added to this oil under argon at 0 °C, followed by the dropwise addition of methanesulfonyl chloride (15.48 mL, 22.91 g, 200 mmol) in dry dichloromethane (75 mL). Once addition was complete, the reaction was left to stir for 5 h at room temperature. A further volume of dichloromethane (300 mL) was added to the reaction and the organic solution was partitioned with satd. aq. $NaHCO_3$ (2 × 300 mL). The organic phase was then partitioned with satd. aq. NaCl (300 mL), dried with anhydrous sodium sulfate and concentrated in vacuo to give a brown oil. This oil was dried by azeotropic distillation several times with toluene. The brown oil was dissolved in dimethylformamide (150 mL) and sodium azide (32.51 g, 500 mmol) was added. The reaction was left to stir for four days after which time it was concentrated in vacuo and then redissolved in diethyl ether (1000 mL). The ethereal solution was filtered and the filtrate was concentrated in vacuo to give a brown oil. This oil was purified by normal-phase silica gel chromatography, eluting with diethyl ether only, to yield the title compound **20** as a yellow oil (33.4 g, 76% from **4**). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.37 (t, J = 5.1 Hz, 2 H, $CH_2CH_2N_3$), 3.60–3.69 [m, 26 H, $(CH_2CH_2O)_6CH_2CH_2N_3$], 4.56 (s, 2 H, $C_6H_5CH_2O$), 7.22–7.35 (m, 5 H, $OCH_2C_6H_5$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 50.7, 69.4, 70.0, 70.57, 70.59, 70.64, 70.7, 73.2, 127.6, 127.7, 128.4, 138.4 (signals superimposed) ppm. IR (oil): $\tilde{\nu}$ = 3010 (aryl-H), 2867 (C–H), 2106 (N_3), 1453 (C=C) cm^{-1}. MS (positive ES): m/z (%) = 464 (100) [M + Na]$^+$, 436 (4) $[C_{21}H_{35}O_7N + Na]^+$. HRMS (positive ion FAB): calcd. for [M + H]$^+$ 442.2553; found 442.2566.

20-(Phenylmethyloxy)-3,6,9,12,15,18-hexaoxaeicosylamine (21): Triphenylphosphane (23.3 g, 88.9 mmol) was added to 1-azido-20-(phenylmethyloxy)-3,6,9,12,15,18-hexaoxaeicosane (**20**) (32.7 g, 74.1 mmol) in tetrahydrofuran (200 mL). The solution was stirred for 3 h at room temperature, then water (5.40 g, 296 mmol) was added. The reaction was then stirred at room temperature for 48 h, after which the reaction was concentrated in vacuo. Water (500 mL) was added to the remaining oil. The reaction was then filtered to remove the precipitate, the precipitate being washed twice with water (2 × 150 mL). The filtrate was combined and acidified to pH 2 with concd. hydrochloric acid. This solution was partitioned four times with diethyl ether (4 × 300 mL). The aqueous solution was then adjusted to pH 11 by addition of solid Na_2CO_3 and also saturated with NaCl. The resulting solution was partitioned four times with dichloromethane (4 × 300 mL). The organic fractions were combined, dried with anhydrous sodium sulfate and concentrated in vacuo to yield **21** as a colourless oil, which required no further purification (29.8 g, 97%). ^{1}H NMR (300 MHz, DMSO): δ = 2.65 (t, J = 5.8 Hz, 2 H, CH_2NH_2), 3.36 (t, J = 5.8 Hz, 2 H, $CH_2CH_2NH_2$), 3.49–3.61 [m, 24 H, $C_6H_5CH_2O(CH_2CH_2O)_6$], 4.51 (s, 2 H, $C_6H_5CH_2O$), 7.27–7.39 (m, 5 H, $C_6H_5CH_2O$) ppm. ^{13}C NMR (75 MHz, DMSO): δ = 42.3, 70.1, 70.5, 70.7, 73.0, 74.1, 128.3, 128.4, 129.1, 139.4 (signals superimposed) ppm. IR (oil): $\tilde{\nu}$ = 3377, 3313 (N–H), 3086, 3062, 3028 (aryl-H), 2865 (C–H), 1583 (N–H bend and C=C) cm^{-1}. MS (positive ES): m/z (%) = 438 (12) [M + Na]$^+$, 416 (99) [M + H]$^+$, 524 (13) $[C_7H_7]^+$. HRMS (positive ESI): calcd. for [M + H]$^+$ 416.26428; found 416.26396.

20-(Fluoren-9-ylmethyloxycarbonylamino)-3,6,9,12,15,18-hexaoxaeicosan-1-ol (22): A solution of 1-amino-20-(phenylmethyloxy)-3,6,9,12,15,18-hexaoxaeicosane (**21**) (29.8 g, 71.6 mmol) in dry diethyl ether (200 mL) under argon was cooled to –78 °C. Ammonia was condensed into this solution until the volume of the solution had almost doubled (ca. 400 mL). Sodium pellets were added to the solution under argon at –78 °C until a dark-blue colour persisted. The reaction was warmed from –78 to –30 °C and then cooled back to –78 °C. A saturated methanolic solution of ammonium chloride was slowly added to the reaction at –78 °C under argon until the solution was no longer blue in colour. The reaction was then warmed to room temperature and once most of the ammonia had evaporated, the reaction was concentrated in vacuo. Water (400 mL) was added to the remaining residue and the pH was adjusted to 4 with concd. hydrochloric acid. The pH of the solution was then adjusted to 7 by addition of solid sodium hydrogen carbonate. Once the solution had been neutralised, $NaHCO_3$ (9.02 g, 107.4 mmol) was again added, followed by dioxane (200 mL). This solution was cooled to 0 °C and fluoren-9-ylmethyl chloroformate (27.78 g, 107.4 mmol) in dioxane (250 mL) was slowly added. Once addition was complete the reaction was warmed to room temperature and stirred for 24 h. The solution was then acidified to pH 6 by addition of solid citric acid and then concentrated in vacuo to remove dioxane. The remaining aqueous solution was partitioned three times with chloroform (3 × 350 mL). The organic fractions were combined, dried with anhydrous sodium sulfate and concentrated in vacuo to give a yellow oil. This oil was purified by normal-phase silica gel chromatography, eluting initially with ethyl acetate only and then gradually changing to ethyl acetate/methanol (4:1), to yield **22** as a yellow oil (38.0 g, 97%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.88 (br. s, 1 H, OH), 3.39 (q, J = 5.2 Hz, 2 H, CH_2NHFmoc), 3.55–3.63 [m, 34 H, $HOCH_2(CH_2OCH_2)_6$], 3.70 (m, 2 H, $HOCH_2$), 4.22 (t, J = 6.9 Hz, 1 H, $NHCOOCH_2CH$), 4.41 (d, J = 6.9 Hz, 2 H, $CH_2NHCOCH_2$), 5.57 (t, J = 5.2 Hz, 1 H, NH), 7.31 (t, J = 7.4 Hz, 2 H, Fmoc aryl), 7.39 (t, J = 7.4 Hz, 2 H, Fmoc aryl), 7.61 (d, J = 7.4 Hz, 2 H, Fmoc aryl), 7.76 (d, J = 7.4 Hz, 2 H, Fmoc aryl) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 41.0, 47.3, 61.7, 66.5, 70.1, 70.3, 70.6, 72.6, 120.0, 125.1, 127.1, 127.7, 141.3, 144.0, 156.6 (signals superimposed) ppm. IR (oil): $\tilde{\nu}$ = 3326 (O–H and N–H), 3041, 3020 (aryl-H), 2884 (C–H), 1672 (C=O stretch and N–H bend), 1452

(C=C) cm^{-1}. MS (positive ES): *m/z* (%) = 548 (9) [M + H]$^+$, 178 (35) [$C_{14}H_{11}$]$^+$. HRMS (FAB, NOBA matrix): calcd. for [M + H]$^+$ 548.2859; found 548.2868.

20-(Fluoren-9-ylmethyloxycarbonylamino)-3,6,9,12,15,18-hexaoxaeicosanoic Acid (Fmoc-Haa7, 2): Chromium trioxide (10.50 g, 105 mmol) in sulfuric acid (1.5 M, 210 mL, 315 mmol) was very slowly added to vigorously stirring 20-(fluoren-9-ylmethyloxycarbonylamino)-3,6,9,12,15,18-hexaoxaeicosan-1-ol (**22**) (19.2 g, 35.0 mmol) in acetone (500 mL) at 0 °C. Once addition was complete the reaction was left to warm to room temperature and to stir for 24 h. After this time water (1000 mL) was added to the reaction along with reversed-phase silica gel (250 g). The reaction mixture was concentrated in vacuo until the volume had decreased by approximately one quarter and more water (500 mL) was then added. The reaction mixture was once again concentrated in vacuo until no acetone could be detected. The mixture was then filtered to recover the reversed-phase silica gel which was washed with copious amounts of water until the silica gel was almost colourless. The reversed-phase silica gel was then washed with acetonitrile (4 × 400 mL) and chloroform (3 × 300 mL). The organic fractions were combined and concentrated in vacuo to give a green oil. This oil was purified by normal-phase silica gel chromatography, eluting with a gradient [chloroform to chloroform/methanol (95:5) to chloroform/methanol/acetic acid (85:10:5)] to yield **2** as a yellow oil (14.2 g, 71%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.38 (m, 2 H, FmocHNC*H*$_2$), 3.59–3.62 [m, 22 H, FmocHNCH$_2$(C*H*$_2$OC*H*$_2$)$_5$], 4.04 (s, 2 H, CH$_2$OC*H*$_2$CO$_2$H), 4.22 (t, *J* = 6.7 Hz, 1 H, NHCOOCH$_2$C*H*), 4.42 (d, *J* = 6.7 Hz, 2 H, NHCOOC*H*$_2$), 5.73 (br. s, 1 H, N*H*), 7.31 (t, *J* = 7.4 Hz, 2 H, Fmoc aryl), 7.41 (t, *J* = 7.4 Hz, 2 H, Fmoc aryl), 7.61 (d, *J* = 7.4 Hz, 2 H, Fmoc aryl), 7.77 (d, *J* = 7.4 Hz, 2 H, Fmoc aryl) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 41.0, 47.5, 66.6, 69.98, 70.02, 70.1, 70.3, 120.1, 125.3, 127.3, 127.9, 141.5, 144.2, 156.9, 172.8 ppm. IR (oil): $\tilde{\nu}$ = 3333 (O–H and N–H), 2871 (C–H), 2550 (COO–H), 1717 (C=O), 1605 (C=O), 1538 (N–H) cm^{-1}. MS (positive ion FAB): *m/z* (%) = 584 (58) [M + Na]$^+$, 562 (2) [M + H]$^+$, 179 (45) [$C_{14}H_{11}$]$^+$, 165 (6) [$C_{13}H_9$]$^+$. MS (positive ES): *m/z* (%) = 600 (27) [M + K]$^+$, 584 (99) [M + Na]$^+$. HRMS (FAB, NOBA matrix): calcd. for [M + Na]$^+$ 584.2472; found 584.2495.

Peptide Synthesis: The solid support used for SPPS was a NovaSyn-TGT resin preloaded with *N*-α-Fmoc-glycine. All SPPS reactions were carried out at room temperature.

Peptide–Resin Cleavage and Peptide Deprotection. General Procedure: A solution (10 mL) consisting of trifluoroacetic acid (85%), thioanisole (5%), phenol (5%), water (2.5%) and triethylsilane (2.5%) was added to the dry resin-bound peptide (0.110 mmol) in a Merrifield bubbler. The resin-bound peptide in solution was left to agitate for 10 min by bubbling with a stream of nitrogen. After 10 min, the solution was drained into a flask. Another volume of the solution (10 mL) was then added to the resin-bound peptide and again bubbling with nitrogen was recommenced for 10 min. The solution was drained into the flask and the above procedure was repeated four more times. Once this was complete, the content of the flask was stirred at room temperature under nitrogen for 6 h. After 6 h, the solution was concentrated in vacuo and diethyl ether/hexane (1:1, 50 mL) was added to the residue that remained. The organic solution was carefully decanted to retain the solid precipitate that had formed, which was then dried under vacuum. The solid was then redissolved in water, degassed under vacuum and freeze-dried.

Disulfide Bridge Formation. General Procedure: The peptide was dissolved in degassed water to give a final concentration of around 0.25 mg/mL. The solution was stirred at room temperature exposed to the atmosphere for one week. After one week the reaction was concentrated in vacuo, the remaining residue was redissolved in degassed water and freeze-dried.

Synthesis of the Fully Protected Peptide–Resin 23: The sequence Fmoc-Gly-Ala-Cys(Trt)-Arg(Pbf)-Arg(Pbf)-Glu(O*t*Bu)-Thr(*t*Bu)-Ala-Trp(Boc)-Ala-Cys(Trt)-Gly-OTGT was assembled on the MilliGen 9050 using standard procedures (deblock: 30% piperidine in DMF; coupling: 0.6 M DIPCI in DMF/0.6 M HOBt in DMF; auxiliary wash: 0.3 M NaI in DMF; wash: DMF/DCM, 3:2).

Peptide A: Peptide A was synthesised using standard procedures from **23**, then cleaved from the resin and oxidised using the procedures outlined above and purified by HPLC.

Peptide B: Acid **1** was added to the fully protected peptide–resin **23** and coupled with 0.6 M TBTU and 0.6 M HOBt in DMF/1.0 M DIPEA. The Fmoc deprotection time was extended by 10 min and the recycle time also by 10 min. The remainder of the sequence was then also assembled on the MilliGen 9050 apparatus using standard procedures, coupling Fmoc-Lys(Boc) with 0.6 M TBTU and 0.6 M HOBt in DMF/1.0 M DIPEA. The peptide was cleaved from the resin and deprotected by the standard procedure. The residue was analysed by MS and was found to contain the desired peptide. The residue was then redissolved in water, degassed under vacuum and freeze-dried. The residue was purified by preparative reversed-phase HPLC using water/acetonitrile (5% MeCN to 20% MeCN in 30 min, flow rate 15 mL/min) as eluent. The retention time of the product was approximately 27 min (H_2O/MeCN, 81:19). MS (positive ES): calcd. 3520.40; found 3518.80 (503.76 [M + 7H]$^+$/7, 587.45 [M + 6H]$^+$/6, 704.69 [M + 5H]$^+$/5, 880.70 [M + 4H]$^+$/4, 1174.04 [M + 3H]$^+$/3). The disulfide bridge was then formed by aerial oxidation following the standard procedure. The residue was then purified by preparative reversed-phase HPLC using water/acetonitrile [5% MeCN to 20% MeCN in 30 min, flow rate 15 mL/min, retention time 29 min (H_2O/MeCN, 80:20)] as the eluent. MS (positive ES): calcd. 3518.38; found 3515.75 (704.12 [M + 5H]$^+$/5, 879.89 [M + 4H]$^+$/4, 1172.92 [M + 3H]$^+$/3).

Peptide C: The resin-bound peptide **23** (0.37 g, 0.055 mmol) was removed from the Millipore synthesiser and pre-swelled with DMF on a Merrifield bubbler under nitrogen. The DMF was removed, DBU/piperidine/DMF (1:1:48, 8 mL) was added to cover the resin-bound peptide and the reaction was agitated by nitrogen bubbling for 10 min. The deprotection solution was then removed and the resin-bound peptide was washed with DMF (2 × 10 mL). A further quantity of DBU/piperidine/DMF (1:1:48, 8 mL) was again added and bubbling was recommenced for 10 min, followed by washing with DMF. This procedure was repeated a further three times. After the final deprotection, the resin-bound peptide was thoroughly washed with DMF (50 mL). 20-(Fluoren-9-ylmethyloxycarbonylamino)-3,6,9,12,15,18-hexaoxaeicosanoic acid (**2**) (0.123 g, 0.22 mmol), HATU (0.083 g, 0.22 mmol) and HOAt (0.029 g, 0.22 mmol) were dissolved in the minimum quantity of DMF (5 mL). *N*,*N*-Diisopropylethylamine (0.096 mL, 0.0711 g, 0.55 mmol) was added to this solution and the resulting solution was immediately added to the resin-bound peptide. The reaction was agitated by bubbling with nitrogen for 1 h. The reagent solution was removed and the resin-bound peptide was washed with DMF (2 × 20 mL). DBU/piperidine/DMF (1:1:48, 8 mL) was added to cover the resin-bound peptide and the reaction was agitated by nitrogen bubbling over 10 min. The deprotection solution was then removed and the resin-bound peptide was washed with DMF (2 × 10 mL). A further quantity of DBU/piperidine/DMF (1:1:48, 8 mL) was again added and bubbling was recommenced for

10 min, followed by washing with DMF. This procedure was repeated a further three times. After the final deprotection, the resin-bound peptide was thoroughly washed with DMF (50 mL). Fmoc-Lys(Boc)-OH (0.103 g, 0.22 mmol), HATU (0.083 g, 0.22 mmol) and HOAt (0.029 g, 0.22 mmol) were dissolved in the minimum quantity of DMF (5 mL). *N*,*N*-Diisopropylethylamine (0.096 mL, 0.071 g, 0.55 mmol) was added to this solution and the resulting solution was immediately added to the resin-bound peptide. The reaction was agitated by bubbling with nitrogen for 1 h. The reagent solution was removed and the resin-bound peptide was washed with DMF, dichloromethane, and diethyl ether, then dried under vacuum. A small quantity of the resin-bound peptide (≈ 40 mg) was cleaved and deprotected by the standard procedure. The residue recovered from cleavage/deprotection was analysed by mass spectroscopy and was found by mass spectrometry to contain almost exclusively the desired product. MS (positive ES): calcd. 1952.24; found 1953.01 (652.1 [M + 3H]$^+$/3, 977.4 [M + 2H]$^+$/2). The remaining resin-bound peptide was reloaded onto the reaction column in DMF and the remainder of the sequence was assembled on the MilliGen 9050 synhesiser using standard procedures, coupling Fmoc-Lys(Boc)-OH with 0.6 M TBTU and 0.6 M HOBt in DMF/1.0 M DIPEA. The peptide was cleaved from the resin and deprotected using the general procedure. The residue was analysed by MS and was found to contain the desired peptide. The residue was then redissolved in water, degassed under vacuum and freeze-dried. The residue was then purified by preparative reversed-phase HPLC using water/acetonitrile [5% MeCN to 25% MeCN in 30 min then 100% MeCN in 10 min, flow rate 15 mL/min, retention time 20 min (H_2O/MeCN, 83:17)] as eluent. MS analysis of the peptide recovered from HPLC purification showed that the peptide had been purified and recovered: MS (positive ES): calcd. 3652.61; found 3652.56 (457.6 [M + 8H]$^+$/8, 522.8 [M + 7H]$^+$/7, 609.7 [M + 6H]$^+$/6, 731.6 [M + 5H]$^+$/5). The disulfide bridge was then formed by aerial oxidation following the standard procedure. The residue was then purified by preparative reversed-phase HPLC using water/acetonitrile [5% MeCN to 25% MeCN in 30 min then 100% MeCN in 10 min, flow rate 15 mL/min, retention time 22 min (H_2O/MeCN, 81:19)] as eluent. MS analysis of the peptide recovered from HPLC purification showed that the peptide had been successfully oxidised. MS (positive ES): calcd. 3650.60; found 3651.01 (457.4 [M + 8H]$^+$/8, 522.6 [M + 7H]$^+$/7, 609.5 [M + 6H]$^+$/6, 731.1 [M + 5H]$^+$/5).

Peptide D: Acid **3** was added to the fully protected peptide–resin **23** and coupled with 0.6 M TBTU and 0.6 M HOBt in DMF/1.0 M DIPEA. The Fmoc deprotection time was extended by 10 min and the recycle time also by 10 min. The remainder of the sequence was then also assembled on the MilliGen 9050 synthesiser using standard procedures, coupling Fmoc-Lys(Boc)-OH with 0.6 M TBTU and 0.6 M HOBt in DMF/1.0 M DIPEA. The peptide was cleaved from the resin and deprotected by the standard procedure. The residue was analysed by MS and found to contain the desired peptide. The residue was then redissolved in water, degassed under vacuum and freeze-dried. The residue was purified by preparative reversed-phase HPLC using water/acetonitrile (10% MeCN to 20% MeCN in 40 min, flow rate 15 mL/min, retention time 36 min (H_2O/MeCN, 81:19) as eluent. MS analysis of the peptide recovered from HPLC purification showed that the peptide had been purified and recovered. MS (positive ES): calcd. 3740.71; found 3742.71 (535.85 [M + 7H]$^+$/7, 624.83 [M + 6H]$^+$/6, 749.40 [M + 5H]$^+$/5, 936.49 [M + 4H]$^+$/4). The disulfide bridge was then formed by aerial oxidation following the standard procedure. The residue was then purified by preparative reversed-phase HPLC using water/acetonitrile [10% MeCN to 15% MeCN in 5 min then 20% MeCN in 35 min, flow rate 15 mL/min, retention time 29 min (H_2O/MeCN, 83:17)]. MS analysis of the peptide recovered from HPLC purification showed that the peptide had been successfully oxidised. MS (positive ES): calcd. 3738.69; found 3740.26 (468.56 [M + 8H]$^+$/8, 535.34 [M + 7H]$^+$/7, 624.37 [M + 6H]$^+$/6, 748.98 [M + 5H]$^+$/5).

Peptide E: Two equiv. of Fmoc-protected Haa4 (**1**) were added sequentially to the fully protected peptide–resin **23** and coupled with 0.6 M TBTU and 0.6 M HOBt in DMF/1.0 M DIPEA. The Fmoc deprotection time was extended by 10 min and the recycle time also by 10 min. The remainder of the sequence was then also assembled on the MilliGen 9050 synthesiser using standard procedures, coupling Fmoc-Lys(Boc)-OH with 0.6 M TBTU and 0.6 M HOBt in DMF/1.0 M DIPEA. The peptide was cleaved from the resin and deprotected by the standard procedure. The residue was analysed by MS and was found to contain the desired peptide. The residue was then redissolved in water, degassed under vacuum and freeze-dried. The residue was purified by preparative reversed-phase HPLC using water/acetonitrile [5% MeCN to 20% MeCN in 30 min, flow rate 15 mL/min, retention time 28 min (H_2O/MeCN, 81:19)] as eluent. MS analysis of the peptide recovered from HPLC purification showed that the peptide had been purified and recovered. MS (positive ES): calcd. 3709.05; found 3708.78 (464.72 [M + 8H]$^+$/8, 530.86 [M + 7H]$^+$/7, 619.14 [M + 6H]$^+$/6, 742.75 [M + 5H]$^+$/5, 928.05 [M + 4H]$^+$/4). The disulfide bridge was then formed by aerial oxidation following the standard procedure. The residue was then purified by preparative reversed-phase HPLC using water/acetonitrile [5% MeCN to 20% MeCN in 5 min, flow rate 15 mL/min, retention time 29 min (H_2O/MeCN, 80:20)] as eluent. MS analysis of the peptide recovered from HPLC purification showed that the peptide had been successfully oxidised. MS (positive ES): calcd. 3707.60; found 3705.12 (464.11 [M + 8H]$^+$/8, 530.40 [M + 7H]$^+$/7, 618.63 [M + 6H]$^+$/6, 742.02 [M + 5H]$^+$/5, 927.18 [M + 4H]$^+$/4).

Transfections: The normal human airway epithelial cell line,[59] 1HAEo- (D. Gruenert, University of California, San Francisco), was maintained in Eagle's Minimal Essential Medium (MEM) HEPES modification (Sigma, Poole, U.K.) containing 10% foetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine, as described previously.[60] Neuro-2A cells (ATCC) were maintained in Dulbecco's Modified Eagle Medium (DMEM) Glutamax-1 (Life Technologies, Paisley, U.K.) containing 10% foetal calf serum (FCS) (Sigma, Poole, U.K.), 100 IU/mL penicillin, 100 µg/mL streptomycin, 1% sodium pyruvate and 1% non-essential amino acids (Life Technologies, Paisley, U.K.). AJ3.1 is a mouse embryonic fibroblast line derived from A/J embryos 13.5 days postcoitum.

Lipopolyplex formulations were prepared by mixing the components in the following order: 50 µL of Lipofectin (InVitrogen Ltd, Paisley, U.K., 30 µg/mL in OptiMEM), 70 µL of peptide (at between 0.029 and 0.1 mg/mL in OptiMEM, according to the peptide charge ratio used) and 50 µL of the luciferase reporter plasmid pCILux (40 µg/mL in OptiMEM). Plasmid pCILux was prepared by subcloning a luciferase gene from pGL3 Control (Life Technologies, Paisley, U.K.) into the eukaryotic expression vector pCI (Promega, Southampton, U.K.). The complex was mixed by rapid addition by pipette before diluting in OptiMEM to a final volume of 1.57 mL. The complete growth medium was removed from cells plated at 2×10^4 cells/well overnight in 96-well plates and 200 µL of the complex (0.25 µg of plasmid DNA) was added to each well, leaving minimal time between preparing the complex and adding it to the cells. All transfections were carried out in 6 wells each. The cells were incubated with the complexes for 4 h before replacing with normal media for 24–48 h, after which reporter gene expression was analysed by luciferase assay (Promega, Southampton, U.K.).

Luciferase assays were performed as follows. Cells were washed twice with PBS before the addition of 100 µL of 1 × Reporter Lysis Buffer (Promega, Southampton, U.K.) to the cells for 20 min at 4 °C before freezing at –20 °C for at least 30 min followed by thawing at room temperature (±20 °C). The lysate (20 µL) was transferred at room temperature to a white polystyrene 96-well plate (Porvair Sciences Ltd., Shepperton, U.K.) and the luciferase activity was measured using the Luciferase Assay System (Promega) and a Lucy-1 Luminometer (Anthos Ltd., Saltzburg, Austria). The amount of protein present in each transfection lysate was determined with the Bio-Rad protein assay reagent following the manufacturer's instructions, adding 20 µL from the luciferase test to 200 µL of the reagent diluted to 1 in 5 and incubating at room temperature for 10 min before comparing the OD_{590} to a range of BSA standards. Luciferase activity was expressed as relative light units (RLU) per mg of protein (RLU/mg).

DLS Measurements: The complexes were formulated as described above, but by using the PSVβ plasmid (a 6.9 kilobase plasmid). DLS measurements were performed using a Malvern Zetasizer 3000 instrument (Malvern Instruments Ltd., UK) operating at a wavelength of 633 nm and a power output of 5 mW. All experiments were conducted at a constant temperature of 25 °C and a scattering angle of 90°, and spectra were collected every 180 s for at least 30 min. For broad, irregularly shaped distributions, for which the method of cumulants was inappropriate, the CONTIN "constrained regularisation" program[61,62] was used for data analysis.

Supporting Information (see also the footnote on the first page of this article): ^{13}C NMR spectra of **2**–**7**, **11**, **16**–**18** and **20**–**22**. ES-MS and HPLC data for peptides **B**–**E**.

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